Localization of Ribosomal Protein S1 in the Granular Component of the Interphase Nucleolus and Its Distribution during Mitosis

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ABSTRACT Using antibodies to various nucleolar and ribosomal proteins, we define, by immunolocalization in situ, the distribution of nucleolar proteins in the different morphological nucleolar subcompartments. In the present study we describe the nucleolar localization of a specific ribosomal protein (S1) by immunofluorescence and immunoelectron microscopy using a monoclonal antibody (RS1-105). In immunoblotting experiments, this antibody reacts specifically with the largest and most acidic protein of the small ribosomal subunit (S1) and shows wide interspecies cross-reactivity from amphibia to man. Beside its localization in cytoplasmic ribosomes, this protein is found to be specifically localized in the granular component of the nucleolus and in distinct granular aggregates scattered over the nucleoplasm. This indicates that ribosomal protein S1, in contrast to reports on other ribosomal proteins, is not bound to nascent pre-rRNA transcripts but attaches to preribosomes at later stages of rRNA processing and maturation. This protein is not detected in the residual nucleolar structures of cells inactive in rRNA synthesis such as amphibian and avian erythrocytes. During mitosis, the nucleolar material containing ribosomal protein S1 undergoes a remarkable transition and shows a distribution distinct from that of several other nucleolar proteins. In prophase, the nucleolus disintegrates and protein S1 appears in numerous small granules scattered throughout the prophase nucleus. During metaphase and anaphase, a considerable amount of this protein is found in association with the surfaces of all chromosomes and finely dispersed in the cell plasm. In telophase, protein S1-containing material reaccumulates in granular particles in the nucleoplasm of the newly formed nuclei and, finally, in the re-forming nucleoli. These observations indicate that the nucleolus-derived particles containing ribosomal protein S1 are different from cytoplasmic ribosomes and, in the living cell, are selectively recollected after mitosis into the newly formed nuclei and translocated into a specific nucleolar subcompartment, i.e., the granular component. The nucleolar location of ribosomal protein S1 and its rearrangement during mitosis is discussed in relation to the distribution of other nucleolar proteins.

The current concept of the functional organization of the nucleolus is primarily based on electron microscopy and some information at the nucleic acid level. Thus, the nucleolus is defined as a cluster of transcriptionally active rRNA genes that is associated with structures representing a stockpile of ribosomal precursor particles at various stages of processing and maturation (for reviews see references 1–3). By electron microscopy, three morphologically distinct nucleolar components have been distinguished (for reviews see references 4–6): the fibrillar center(s), the dense fibrillar component, and the granular component. Different functions have been assigned to these nucleolar substructures. Recently, RNA polymerase I, the enzyme responsible for transcription of rRNA genes (for review see reference 7), has been localized in the

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fibrillar centers and the nucleolar organizer regions (NOR)[1] of metaphase chromosomes (8), thus identifying the fibrillar centers as the location of transcriptionally active rRNA genes and as the interphase structure corresponding to the chromosomal NOR. Earlier autoradiographic studies that used short pulse labeling with radioactive uridine have revealed structures containing precursor rRNA in association with the dense fibrillar component (9–11) but the micrographs presented do not exclude a relationship of the label to regions containing fibrillar center material. Results of studies that used prolonged labeling and pulse-chase experiments have been interpreted as indicating that ribosomal precursor particles in advanced stages of maturation are mainly localized in the granular component (11, 12).

Much less information is available on the localization of specific proteins in the different nucleolar subcompartments. Using immunoelectron microscopy on spread preparations of actively transcribed rRNA genes ("Christmas tree" structures, reference 13) Chooi and Leiby (14) have reported that a ribosomal protein (S14) is an "early ribosomal protein" already present in ribonucleoprotein complexes of nascent transcripts. However, the specific in situ localization of this ribosomal protein in the nucleolus or in distinct nucleolar subcompartments is not known (for results of immunofluorescence microscopy see reference 15). In addition, two nonribosomal phosphoproteins (B23, C23) have been described as nucleolar components (reviewed in reference 16). Protein C23 (M_r 110,000), an apparently argyrophilic protein, has been reported to be directly or indirectly associated with rDNA and to be located in the fibrillar centers and dense fibrillar component of nucleoli (17–22). On the other hand, protein B23 (M_r 37,000) is believed to be associated with ribosomal precursor particles and to be located in the granular component and, perhaps, also in the fibrillar component (18, 20–22). Moreover, a structural element of the nucleolus, a protein of M_r 145,000, has been identified as the major component of a karyoskeletal protein filament structure present in the nucleolar cortex (23–25).

Ribosome biogenesis is a complex process which includes the coordinate assembly of ribosomal and nonribosomal proteins with pre-rRNA to form ribosomal precursor particles (reviewed in reference 26). We have used an immunological approach to study the assembly of preribosomes and to relate specific nucleolar functions with distinct nucleolar compartments. In this study, we present the first localization of a ribosomal protein in a nucleolar substructure in situ. By using light and electron microscopic immunolocalization, we show that ribosomal protein S1[2] is specifically located in the granular component of the interphase nucleolus and, during disintegration of the nucleolus in mitosis, becomes transiently associated with the surfaces of the chromosomes. Our results suggest that this ribosomal protein associates with rRNA sequences only late in the assembly process i.e., after translocation of the newly synthesized rRNA into the granular component of the nucleolus.

MATERIALS AND METHODS

Biological Materials: Clawed toads (*Xenopus laevis*) were purchased from the South African Snake Farm (Fish Hoek, South Africa). Chicken, mice, and rats (Sprague Dawley) were obtained from local animal farms. Cell lines derived from human (HeLa), rat (RVF-SM), marsupial (PtK2), and amphibian (XLKE cells, line A6) tissues were grown as described (27, 28). Human colon carcinoma tissue was obtained and frozen as described (29).

Monoclonal Antibodies: Monoclonal antibodies were raised essentially according to the method of Köhler and Milstein (30). BALB/c mice were immunized twice by subcutaneous injection of 100 μg of a ribosome-containing cytoskeletal preparation of griseofulvin-intoxicated mouse liver tissue (31), emulsified, for the first immunization, in complete Freund's adjuvant and for the second immunization, 4 wk later, in incomplete Freund's adjuvant. 3 d before cell fusion the mice were given an intraperitoneal booster injection of 100 μg of antigen solubilized in PBS. Spleen cells were fused with cells of the myeloma line Ag 8.653 at a ratio of 5:1, and hybrid cells were selected in medium containing hypoxanthine, aminopterine, and thymidine. Cell culture supernatants were primarily screened for antibodies by indirect immunofluorescence microscopy using frozen sections through mouse liver, and positive cell lines were subcloned twice by limited dilution.

Immunoglobulin subclasses were determined by double immunodiffusion according to Ouchterlony and Nilsson (32), using double-concentrated cell culture supernatant and subclass-specific antibodies to mouse immunoglobulins (Miles-Yeda, Rehovot, Israel) diluted 1:4 in PBS.

For production of ascites fluids, BALB/c mice were injected intraperitoneally with 0.5 ml Pristane (2,6,10,14-tetramethylpentadecane; Sigma Chemie, Munich, Federal Republic of Germany [FRG]) to facilitate tumor growth. 1 wk later, 0.5–1 × 10^6 hybridoma cells were injected intraperitoneally. Ascites fluid was obtained after 10–14 d under sterile conditions. Tumor cells were removed by centrifugation, and the supernatant containing the specific monoclonal antibody was used for antibody purification. IgM antibodies were purified from ascites fluids by gel filtration by use of a Sephacryl S 300 column (Pharmacia Fine Chemicals, Uppsala, Sweden) and 0.5 M NaCl, 50 mM Tris-HCl (pH 7.5) as elution buffer.

Preparations of Ribosomes: Ribosomes from *Xenopus laevis* ovaries were prepared as described by Ford (33) with some modifications. In brief, ovaries were homogenized in 2 vol of 0.25 M sucrose, 35 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT), 50 mM Tris-HCl (pH 7.6), and the homogenate was centrifuged at 3,500 *g* for 20 min at 4°C. The supernatant was then centrifuged for 15 min at 35,000 *g* to remove the mitochondrial fraction. Crude ribosomes were pelleted from the postmitochondrial supernatant for 3 h at 120,000 *g*. For the preparation of ribosomal subunits, the resulting ribosomal pellet was resuspended in high salt buffer (0.5 M KCl, 10 mM Mg-acetate, 20 mM β-mercaptoethanol, 0.1 mM DTT, 20 mM Tris-HCl, pH 7.6; cf. reference 34), and loaded onto 10–40% sucrose gradients made up in high salt buffer. Gradients were run for 16 h at 23,000 rpm in an SW 27 rotor (Beckman Instruments Inc., Fullerton, CA) at 4°C. For preparation of 80S ribosomes, TBS (0.1 M NaCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 7.4) was used instead of high salt buffer. Gradient fractions of 1.2 ml were collected. Peak fractions were pooled and either precipitated by 15% trichloroacetic acid or pelleted by centrifugation at 170,000 *g* for 4 h.

Rat liver ribosomal subunits were prepared according to the methods described by Sherton and Wool (35) and Blobel and Sabatini (36) with several modifications. Rat liver tissue was chilled several times by incubations in cold (0–4°C) 0.25 M sucrose-TKM buffer (25 mM KCl, 5 mM $MgCl_2$, 0.1 mM DTT, 50 mM Tris-HCl, pH 7.5), squeezed through a metal sieve, and homogenized with a tight-fitting glass-Teflon homogenizer. The suspension was centrifuged for 10 min at 17,000 *g* at 4°C. The postmitochondrial supernatant was layered on a discontinuous sucrose gradient (1.35, 1.6, and 2 M sucrose in TKM) and centrifuged for 26 h at 45,000 rpm in a Beckman 50 Ti.2 rotor at 4°C. The resulting crude ribosomal pellets were resuspended in high salt buffer (0.5 M KCl, 3 mM $MgCl_2$, 20 mM β-mercaptoethanol, 0.1 mM DTT, 20 mM Tris-HCl, pH 7.8), and puromycin (SERVA, Feinbiochemica GmbH & Co., Heidelberg, FRG) was added to a final concentration of 0.1 mM. Ribosomes were incubated for 15 min at 37°C and layered on top of 10–40% sucrose gradients made up in high salt buffer. Conditions for centrifugation and fractionation of gradients were as described above.

Gel Electrophoresis and Immunoblotting: Ribosomal proteins were separated by slab gel electrophoresis in the presence of SDS using the gel and buffer system described by Thomas and Kornberg (37) with acrylamide

[1] *Abbreviations used in this paper:* DTT, dithiothreitol; NOR, nucleolar organizer regions.

[2] *Footnote:* The general nomenclature of McConkey et al. (48) is used. Ribosomal protein S1, the largest protein of the small ribosomal subunit (81), has been described, using SDS PAGE, for *Xenopus laevis* and the rat by Martini and Gould (46). However, in the most recent papers based on two-dimensional gel electrophoresis of acetic acid-extracted ribosomal proteins, the acidic protein S1 has not been resolved. The *Xenopus laevis* protein designated "S1" by Pierandrei-Amaldi and Beccari (55) and Pierandrei-Amaldi et al. (56) is less acidic and smaller (M_r 34,000) than the ribosomal protein S1 of *Xenopus* described in this study and probably corresponds to protein S2 of the catalogue of McConkey et al. (48).

concentrations of 18%. For two-dimensional gel electrophoresis, either nonequilibrium pH-gradient electrophoresis or isoelectric focusing was used in the first dimension (38; for modifications see reference 39). Second-dimension electrophoresis in the presence of SDS was performed on 18% acrylamide gels. Before samples were applied, ribosomal material was digested with 0.1 mg/ml pancreatic RNase (Boehringer GmbH, Mannheim, FRG) for 15 min at 37°C, to remove nucleic acids that might interfere with the migration of the proteins into the gel.

For immunoblotting experiments, polypeptides were electrophoretically transferred from gels to nitrocellulose paper essentially according to Towbin et al. (40). Nitrocellulose was saturated with 1% BSA in 140 mM NaCl, 10 mM Tris-HCl (pH 7.4) and followed by overnight incubation with antibody-containing hybridoma cell culture supernatant at 4°C. After several washes with 1 M NaCl, 10 mM Tris-HCl (pH 7.4), nitrocellulose paper sheets were incubated for 2 h with peroxidase-coupled goat anti-mouse IgG+IgM (Medac, Hamburg, FRG) diluted 1:1,000 in 1% BSA. Nitrocellulose was extensively washed with 1 M NaCl, 10 mM Tris-HCl (pH 7.4), which was followed by several washes in 140 mM NaCl, 10 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100. Specifically bound antibody was visualized by adding 4-chloro-1-naphthol and H_2O_2 as substrate (41).

Immunofluorescence Microscopy: Small pieces of tissue were quick-frozen as described (42). Cryostat sections 5 μm thick were air dried and dehydrated in ice-cold acetone for 10 min. Cultured cells grown on coverslips were fixed for 10 min in −20°C methanol and dipped four times in ice-cold acetone. For some experiments cells were exposed to drugs before fixation. Actinomycin D (SERVA) was added to the culture medium to a final concentration of 5 μg/ml for 4 h.

Fixed cells or frozen sections were incubated with RS1-105 antibody (hybridoma cell culture supernatant or purified IgM) for 30 min and washed several times in PBS. Bound antibody was visualized by 30-min incubation with fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulin (Medac) diluted 1:20 in PBS. After they were washed in PBS, specimens were dipped in ethanol and embedded in Mowiol (Hoechst, Frankfurt, FRG). We took photographs with a Zeiss Photomicroscope III (Zeiss, Oberkochen, FRG) equipped with epifluorescence optics, using 40× and 63× oil immersion objectives. General control experiments were done with cell culture supernatant of non-Ig–producing hybridoma cell lines. For comparative studies and specific controls, antibodies against several other nucleolar (cf. references 8, 24, 25, 43) or nuclear (e.g., references 44 and 45) proteins were used.

Electron Microscopy: For conventional fixation, small pieces of regenerating rat liver tissue (18 h after partial hepatectomy; cf. reference 8) were fixed for 30 min in cold 1.5% glutaraldehyde containing 50 mM KCl, 2.5 mM $MgCl_2$, 50 mM sodium cacodylate buffered to pH 7.2; washed in cacodylate buffer; and postfixed in 2% osmium tetroxide for 2 h. After they were washed in distilled water, tissues were dehydrated through graded ethanol solutions and embedded in Epon 812. Ultrathin sections were cut and stained according to standard procedures.

Normal or actinomycin D-treated cultured cells were washed briefly with PBS, and fixative (2.5% glutaraldehyde buffered with 50 mM sodium cacodylate, pH 7.2) was directly added to the rinsed monolayer for 20 min at 4°C. After several washes in cold cacodylate buffer, cells were postfixed in 2% OsO_4 for 2 h, washed in distilled water, and stained with 0.5% uranyl acetate overnight at 4°C. Cells were scraped from the dish and centrifuged for 5 min at 500 *g*, and the cell pellet was dehydrated in graded ethanol solutions. After embedding in Epon 812, ultrathin sections were obtained and stained according to standard procedures.

Actinomycin D-treated cultured XLKE cells were prepared for immunoelectron microscopy in the following way: Cells were scraped off from the culture dishes and centrifuged for 5 min at 500 *g*. The cell pellet obtained was quick-frozen as described (42), and frozen sections 6 μm thick were cut and air dried.

For electron microscopic immunolocalization, cryostat sections through regenerating rat liver or cell pellets were dehydrated in acetone, air dried, and incubated with the primary antibody (50 μg IgM/ml) for 30 min. In some experiments sections were fixed with 2% paraformaldehyde in PBS for 10 min after incubation with the primary antibody. After washing was done with PBS, the sections were incubated overnight at room temperature with goat anti-mouse immunoglobulin coupled to 5- or 20-nm colloidal gold particles (Janssen Life Sciences, Beerse, Belgium) which was diluted 1:2 in PBS. Specimens were thoroughly washed in PBS, fixed in 2.5% glutaraldehyde, and processed for electron microscopy as described (8). Micrographs were taken with a Zeiss EM 10 electron microscope.

RESULTS

Antibody

Hybridoma clone RS1-105 was picked by routine screening of original hybrids derived from a fusion of myeloma cells with spleen cells of a mouse immunized with a residual preparation of mouse liver. Antibody-producing hybridoma cell line RS1-105 was grown in tissue culture and in ascites form. The antibody subclass was determined as IgM.

Characterization of Antigen

Antibody RS1-105 was tested for reaction with ribosomal proteins from different species using the immunoblotting technique. In Fig. 1*a*, proteins obtained from purified ribosomal subunits of *Xenopus laevis* (lanes *1* and *2*) and rat (lanes *3* and *4*) are shown after separation by one-dimensional gel electrophoresis. The molecular weights (M_r) of the ribosomal proteins ranged from ~8,000 to 45,000 (46; for review see reference 47). After incubation with antibody RS1-105 and peroxidase-coupled second antibodies, a strong and specific reaction with one polypeptide of the small ribosomal subunit from both species was recognized (Fig. 1*b*). The

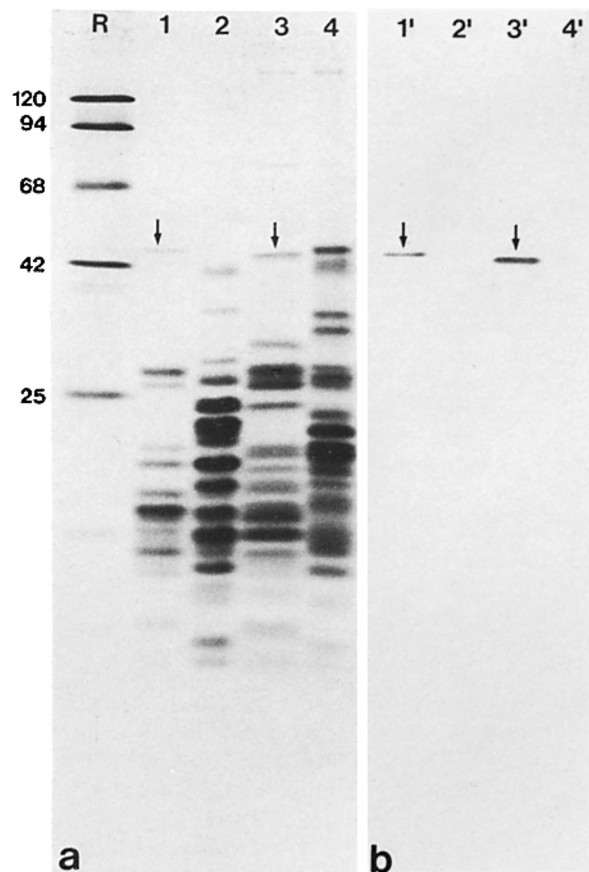


FIGURE 1 One-dimensional gel electrophoresis of ribosomal proteins and identification of the polypeptide reacting with RS1-105 antibody by the immunoblotting technique. (*a*) Coomassie Blue staining of proteins from purified ribosomal subunits isolated from *Xenopus laevis* ovary (lane *1*, small subunit; lane *2*, large subunit) and rat liver (lane *3*, small subunit; lane *4*, large subunit). Ribosomal protein S1 is indicated by arrows. Reference proteins (lane *R*; molecular weights [$\times 10^{-3}$] are indicated) are from top to bottom: β-galactosidase, phosphorylase *a*, BSA, actin, and chymotrypsinogen. (*b*) The polypeptides shown in *a* were transferred to a nitrocellulose filter, probed with antibody RS1-105, and then treated with peroxidase-coupled anti-mouse Ig. The antibody reacts selectively with a single polypeptide (arrows) of the small ribosomal subunit from *Xenopus* (lane *1'*, M_r 45,000) and rat (lane *3'*, M_r 43,000). Note the absence of antibody binding to proteins of the large ribosomal subunits of both species (lanes *2'* and *4'*).

antibody recognized one polypeptide from *Xenopus* of M_r 45,000 (lane *1'*) and from rat a single polypeptide of M_r 43,000 (lane *3'*). The antibody did not react with proteins from the large ribosomal subunit (lanes *2'* and *4'*). Immunoblotting experiments also revealed the presence of the antigenic polypeptide in intact, undissociated 80S ribosomes (data not shown). According to the proposed nomenclature for ribosomal proteins (48), this protein was identified as S1, the largest protein of the small ribosomal subunit. This observation was confirmed by two-dimensional gel electrophoresis of the proteins from the small (40S) ribosomal subunit and immunoblotting with RS1-105 antibody (Fig. 2, *a* and *b*), which revealed the acidic nature of protein S1, both in rat (Fig. 2, *a* and *a'*) and in *Xenopus* (Fig. 2, *b* and *b'*). S1 is the most acidic protein of the small ribosomal subunit, whereas most of the ribosomal proteins migrate into the basic region of the gel. To determine the isoelectric point of protein S1, we separated ribosomal proteins isolated from rat liver 40S ribosomal subunits by two-dimensional gel electrophoresis using isoelectric focusing in the first dimension (Fig. 2*c*). In the presence of 9.5 M urea, protein S1 was isoelectric at a pH of ~5 (Fig. 2*c*, arrow). Other ribosomal proteins were absent from this gel, because basic proteins were not resolved by this gel system.

Light Microscopic Immunolocalization

Indirect immunofluorescence microscopy using RS1-105 antibody on frozen sections through tissues of different species showed strong and specific staining of cytoplasm and nucleoli. In addition, certain small nucleoplasmic granules were also seen to react with this antibody. In previtellogenic oocytes of *Xenopus laevis* (Fig. 3, *a* and *b*) the amplified nucleoli of the large oocyte nucleus and the ribosome-rich cytoplasm were brightly stained. Similarly, somatic cells of *Xenopus*, such as hepatocytes of liver and Sertoli cells of testis, showed both cytoplasmic and nucleolar fluorescence (not shown). Spermatids and testicular spermatozoa were negative. Moreover, the residual nucleolar structures present in amphibian and avian erythrocytes (43, 49) were not stained by RS1-105 antibody (not shown), in agreement with the absence of rDNA transcription and synthesis of ribosomal precursor particles in these structures (49–52). In mammalian tissues such as mouse liver (Fig. 3, *c* and *d*), nucleoli and cytoplasm were specifically decorated by antibodies to protein S1. Nucleolar and cytoplasmic fluorescence was also observed in tumor cells of a human colon carcinoma (Fig. 3*e*).

The specificity of the RS1-105 antibody was also demonstrated in cultured cells (Fig. 4). For example, we observed strong nucleolar and cytoplasmic fluorescence in cells derived from human (HeLa cells; not shown), rat (RVF-SM cells; Fig. 4, *a* and *b*), rat kangaroo (PtK2 cells; Fig. 4, *c–e*) and amphibian (A_6 cells; not shown) origin. The cytoplasmic staining usually could be resolved into a finely granular fluorescence, probably representing groups of ribosomes (Fig. 4, *b–e*). Again occasional small non-nucleolar fluorescent entities were seen in the nucleoplasm. At higher magnification (Fig. 4*e*) the nucleolus did not appear uniformly stained by antibodies to protein S1. Rather, the fluorescence was enriched in peripheral regions of the nucleolus.

Monoclonal antibody RS1-105 showed a broad cross-reactivity with tissues of all species tested, including amphibia (*Xenopus laevis, Pleurodeles waltlii*), birds (chicken), and mammals (rat kangaroo, mouse, rat, man). This indicates a conservation of the antigenic determinant recognized by RS1-105 antibody during evolution.

Electron Microscopic Immunolocalization

We examined the subnucleolar distribution of protein S1 at the electron microscopic level by using monoclonal antibody RS1-105 in combination with secondary antibodies coupled to colloidal gold. For most of our localization studies hepatocytes of regenerating rat liver were used as they present a relatively loose nucleolonema arrangement of nucleolar substructures characterized by a balanced proportion of granular and fibrillar components (Fig. 5; for other rat hepatoma cells see also reference 1). As shown in a survey micrograph in Fig. 6, the granular component, which is known to contain ribosomal precursor particles in advanced stages of maturation (5, 11), was specifically decorated by RS1-105 antibody. Other nucleolar substructures, such as the dense fibrillar component and the fibrillar centers, were not labeled. In Fig. 7*a* the specific localization of protein S1 in the granular component of the nucleolus is shown at higher magnification, in comparison with the specific labeling of the dense fibrillar component by antibody No-114 (Fig. 7*b*), which reacts with a structural nucleolar protein component of M_r 180,000 (43). This differential binding of the two antibodies demonstrates that both nucleolar components are accessible for antibodies.

The only extranucleolar structure that carried antibody label, i.e., gold particles, were distinct, small (0.1–0.3 μm) aggregates of granular appearance (Fig. 8). These S1-positive entities were relative scarce but were found to occur scattered throughout the nucleoplasm of most nuclei examined (Fig. 8). They obviously correspond to the small nucleoplasmic fluorescent entities described above (Figs. 3 and 4).

Localization of Ribosomal Protein S1 in Segregated Nucleoli

Segregation of nucleolar compartments can be induced experimentally by exposing cells to actinomycin D, a strong transcription inhibitor (1, 53, 54). For example, actinomycin-treated A_6 cells of *Xenopus laevis* showed the characteristic segregation of the nucleolus into a phase-dark and a phase-light hemisphere (Fig. 9, *a* and *b*). Antibodies to ribosomal protein S1 stained selectively the phase-dark hemisphere (Fig. 9*a'*), corresponding to the granular component of segregated nucleoli. The dense fibrillar component, which appeared light in phase-contrast optics, was not stained by the antibody to protein S1 but was strongly stained by the antibody against the nucleolar M_r 180,000 protein (Fig. 9*b'*) which is known to be localized in the dense fibrillar component of the nucleolus (43; see also Fig. 7*b*). Electron microscopy of conventionally fixed, actinomycin-treated cells revealed the typical ultrastructural features of the segregated nucleolar hemispheres (Fig. 9*c*). Besides the granular and the dense fibrillar component, a third component was frequently observed in these segregated nucleoli, which appeared as a cap-like crescent at the periphery of the dense fibrillar component and might represent an inactivated form of the fibrillar center. Electron microscopic immunolocalization showed that in such segregated nucleoli the protein S1-containing material was exclusively located in the granular hemisphere (Fig. 9*d*).

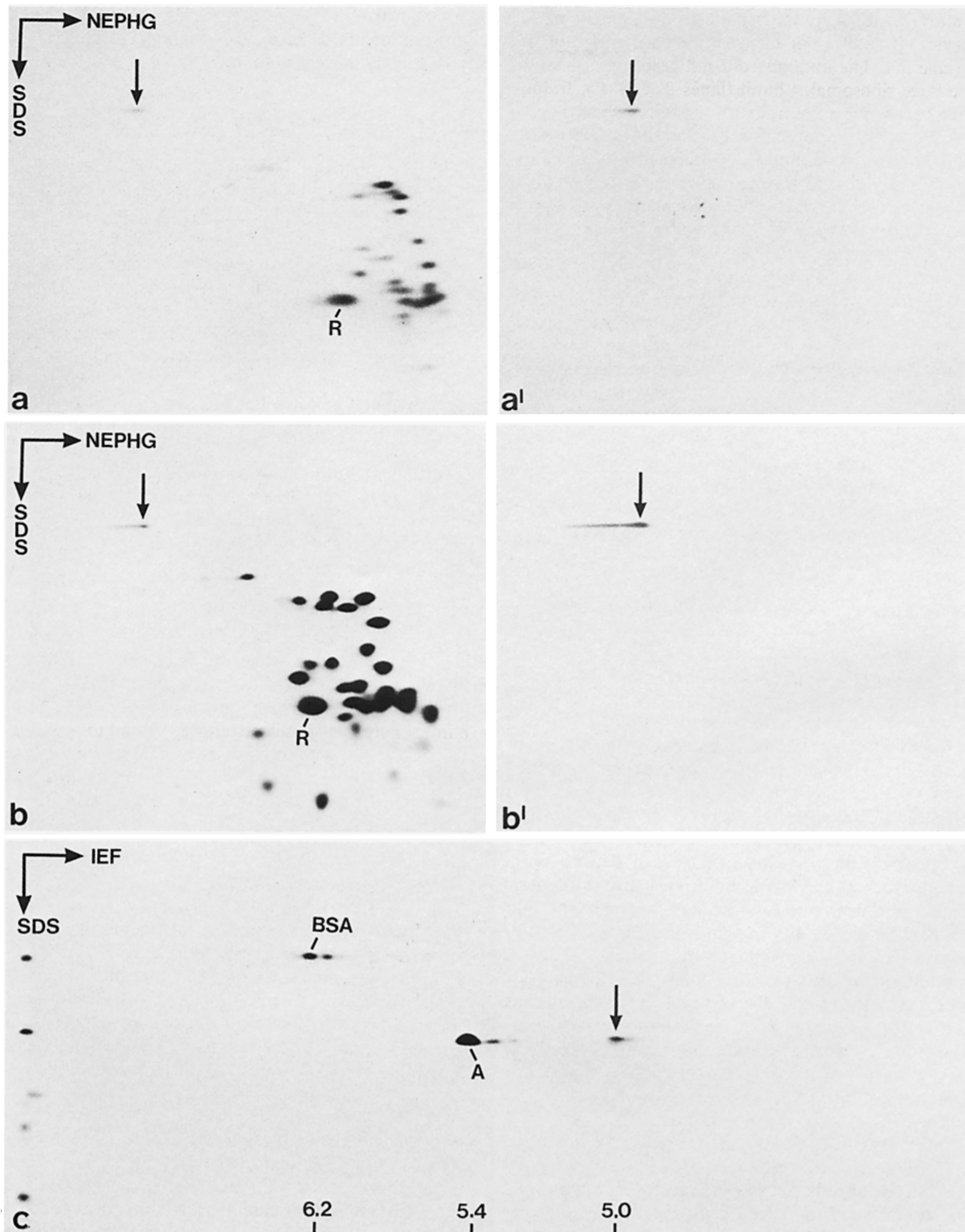


FIGURE 2 Characterization of the antigen recognized by RS1-105 antibody by two-dimensional gel electrophoresis and immunoblotting. (*a* and *b*) Coomassie Blue staining of the proteins of the small ribosomal subunit from rat liver (*a*) and *Xenopus laevis* ovary (*b*) separated by two-dimensional gel electrophoresis (first dimension: nonequilibrium pH-gradient; second dimension: 18% acrylamide, electrophoresis in the presence of SDS). Protein S1 is indicated by arrows; *R* denotes the pancreatic ribonuclease added which also serves as a basic reference protein. (*a'* and *b'*) Immunoblots (same technique as described in Fig. 1*b*), corresponding to the gels shown in *a* and *b*, which demonstrate the specific reaction of RS1-105 antibody with protein S1 (arrows). Note the acidic nature of protein S1. (*c*) Coomassie Blue staining of the proteins of the small ribosomal subunit from rat liver separated by two-dimensional gel electrophoresis using isoelectric focusing (*IEF*) in the first dimension, followed by SDS PAGE (18%) in the second dimension. BSA and actin (*A*) had been added as reference proteins. Only the acidic protein S1 (arrow; isoelectric point [*IEP*] at approximate pH 5) can be identified as basic ribosomal proteins do not migrate into the gel under these specific conditions. Isoelectric points of BSA, actin, and protein S1 are indicated at the bottom of the figure.

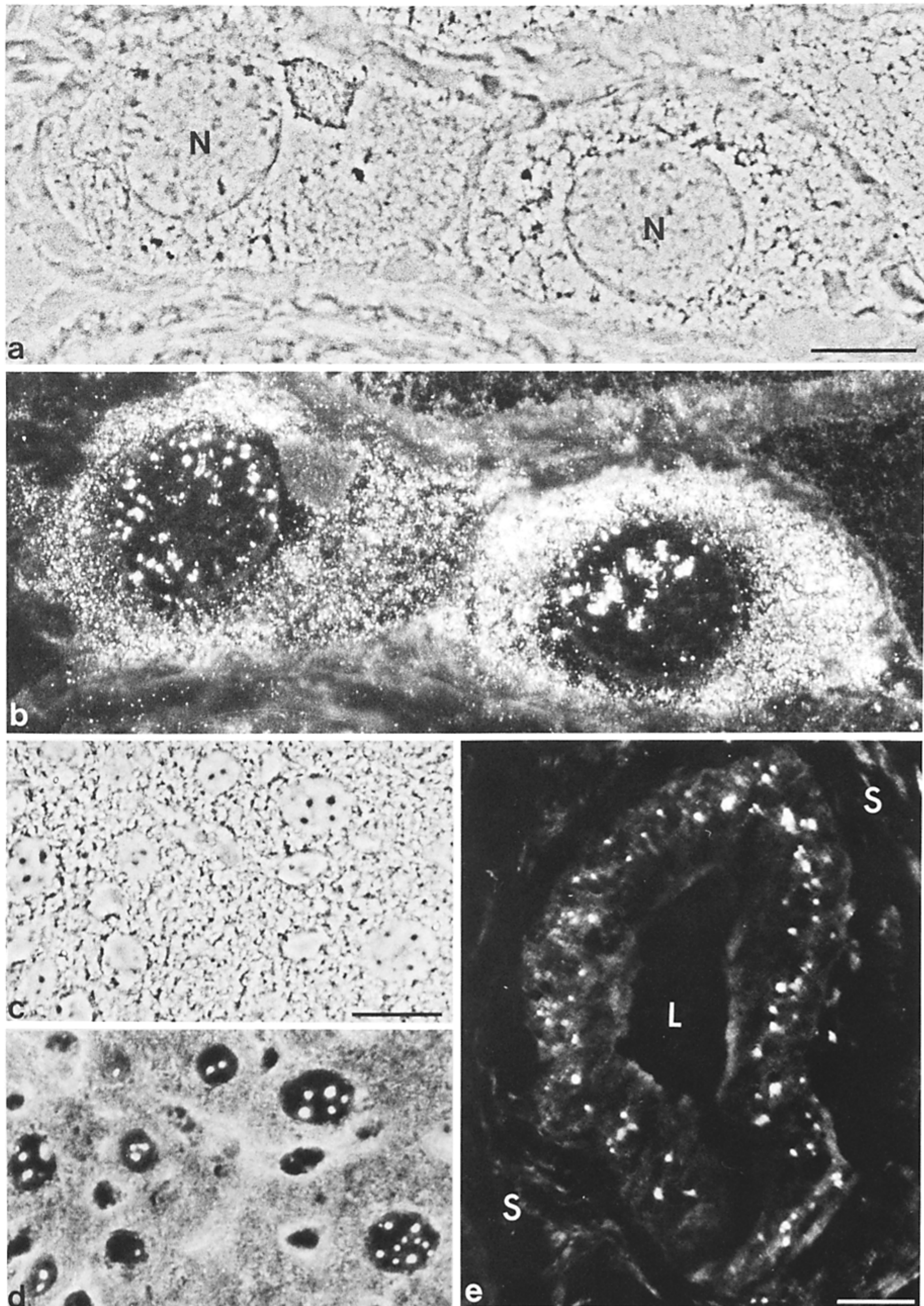


FIGURE 3 Immunofluorescence microscopy of antibody against ribosomal protein S1 on frozen sections of amphibian ovary and mammalian tissues. Previtellogenic oocytes of *Xenopus laevis* are shown in phase-contrast (*a*) and epifluorescence optics (*b*) after incubation with RS1-105 antibody. The cytoplasm and the numerous amplified nucleoli present in the large nuclei (*N*) are brightly fluorescing whereas the nucleoplasm appears negative. In mouse liver tissue (*c*) (phase-contrast), the antibody binds to the nucleoli and to diverse cytoplasmic structures that are probably the ribosomes (*d*). (*e*) Section through a human colonic adenocarcinoma after incubation with the antibody RS1-105, showing strong reaction in nucleoli and diffuse staining in the cytoplasm. Note the large size of the nucleoli of the tumor cells. *L*, lumen of the adenoid structures of the tumor; *S*, stroma. Bars, 20 μm. (*a* and *b*) × 880; (*c* and *d*) × 740; (*e*) × 640.

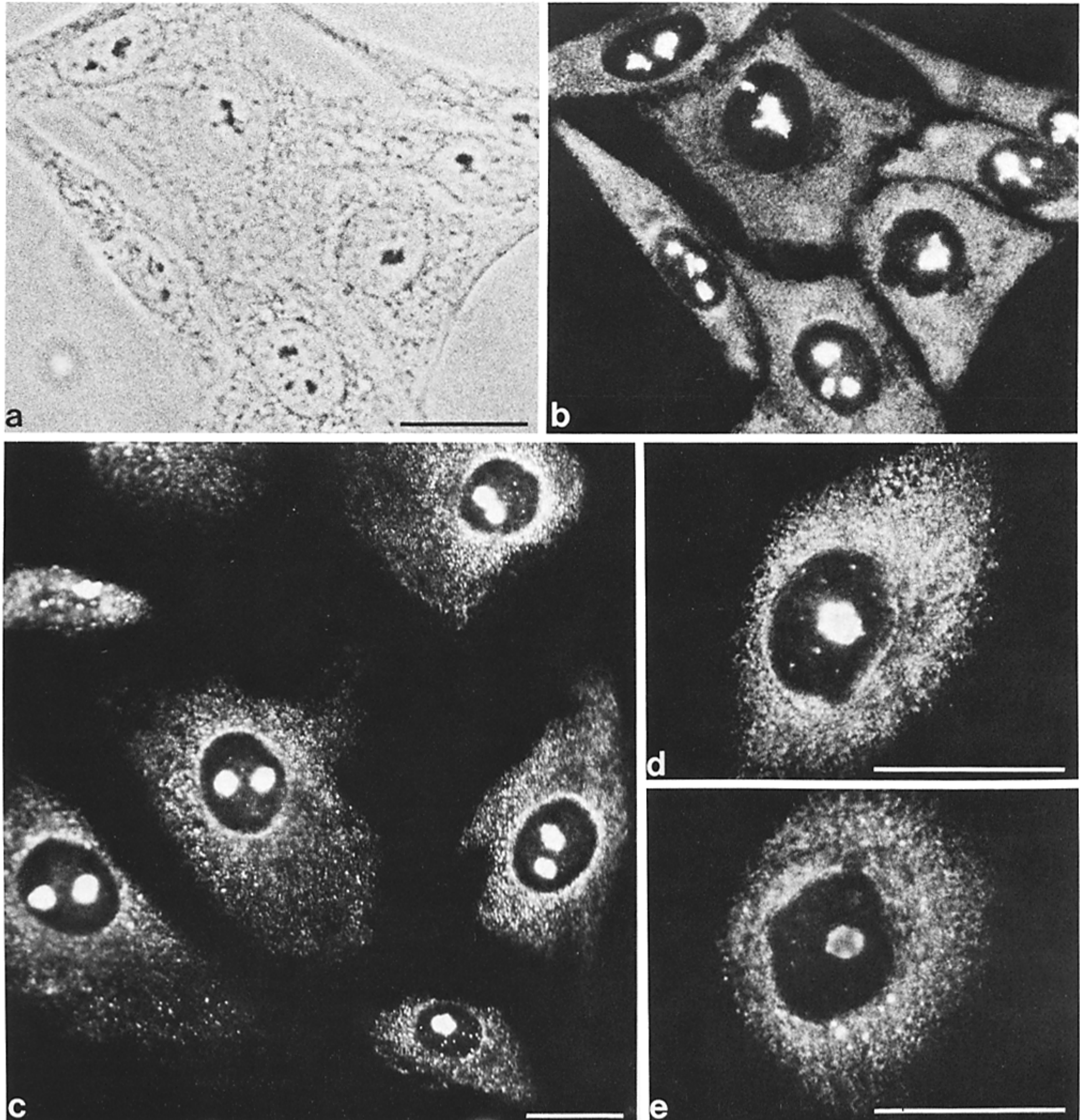


FIGURE 4 Immunofluorescence microscopical localization of ribosomal protein S1 on mammalian cell cultures. Rat (RVF-SM) cells (*a*, phase-contrast; *b*, epifluorescence optics) stained with monoclonal antibody RS1-105 show typical finely punctate cytoplasmic and massive nucleolar staining. The fluorescent nucleolar material, i.e., granular component containing protein S1, occupies a larger area than the phase-contrast dense nucleolar structures, which shows that the nucleolus extends beyond the phase-contrast-dense "core." Rat kangaroo (PtK2) cells (*c–e*) are stained in a similar fashion. In addition, sparse small entities are recognized in the nucleoplasm (*c* and *d*) at higher magnification. At high contrast, one sees that the fluorescence is not uniformly distributed throughout the nucleolus but is more concentrated in the peripheral regions (*e*). Bars, 20 μm. (*a* and *b*) × 1,000; (*c*) × 730; (*d* and *e*) × 1,500.

Distribution of Ribosomal Protein S1 during Mitosis

The cellular distribution of ribosomal protein S1 during mitosis was remarkable. In early mitotic prophase (Fig. 10, *a* and *a'*), the still compact nucleoli, several variously sized (0.2–2 μm diam) nuclear granules, and the cytoplasmic ribosomes were stained by antibody RS1-105. In prometaphase, metaphase, and anaphase (Fig. 10, *b'–d'*), a strong fluorescence was noted on the surfaces of the condensed chromosomes, in addition to a diffuse cytoplasmic staining. It should be noted that this chromosomal association is not restricted

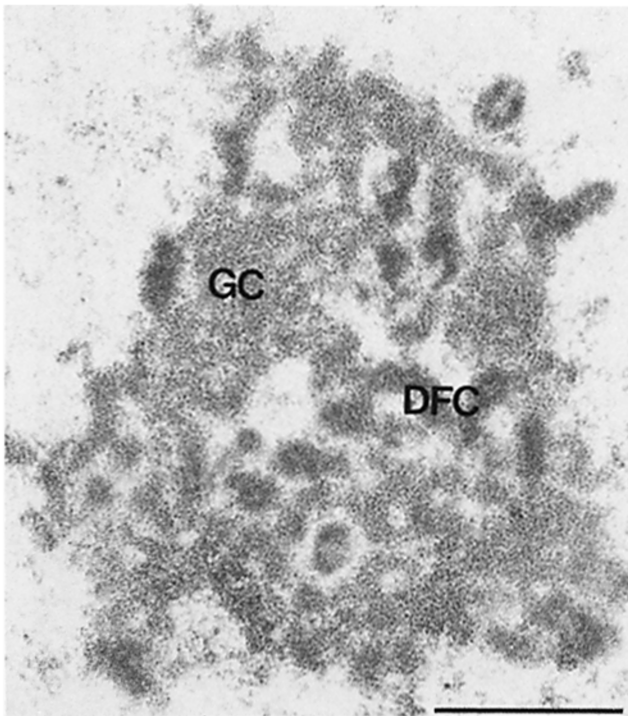


FIGURE 5 Electron micrograph of a nucleolus of regenerating rat liver after standard fixation. Note the loose arrangement of the nucleolar components, typical of a nucleolonema organization. *GC*, granular component; *DFC*, dense fibrillar component. Bar, 1 μm. × 25,000.

to the NOR-containing chromosomes. Moreover, distinct fluorescent granules appeared to be scattered over the cell plasm, often at a considerable distance from the spindle apparatus (Fig. 10, *c′* and *d′*). In late telophase (Fig. 10, *e* and *e′*) and early interphase, a substantial amount of protein S1 was reaccumulated into distinct granules distributed throughout the interior of the forming daughter nuclei and in the reassembling nucleoli. Subsequently, in slightly more advanced interphase, most of the protein S1 antibody binding was again seen in the nucleoli, which indicates that the majority of the scattered nucleoplasmic protein S1-containing granules of early interphase had been amalgamated into the granular components of the nucleoli. Throughout all stages of mitosis, some diffuse staining was seen in the cytoplasm that probably corresponded to the distribution of mature ribosomes. Comparative immunofluorescence studies indicated that the mitotic redistribution pattern of the nucleolar form of ribosomal protein S1 was different from that of RNA polymerase I, used as a marker of transcriptional complexes and fibrillar centers, which remains, to a considerable level, in association with the nucleolar organizer regions of mitotic chromosomes (data not shown; reference 8). The mitotic distribution of the nucleolar ribosomal protein S1 was also different from the behavior of the acidic M_r 180,000 protein, which served as a marker of the dense fibrillar component. Specifically, the M_r 180,000 protein was evenly distributed throughout the cell plasm of metaphase cells and did not show accretion on chromosomal surfaces, nor did it transiently appear in distinct nucleoplasmic granules of the size range of the protein S1-containing particles (43).

DISCUSSION

Our results present the first case of a localization of a ribosomal protein in a distinct structural component of the nucleolus in situ, in this instance the granular component (pars granulosa), where it probably is included in preribosomal particles. All ribosomal proteins examined so far are products of translation from different mRNAs and their synthesis does not involve proteolytic processing of precursor molecules (55; for further references see reference 56). Therefore, it seems justified to assume that the primary structures of ribosomal proteins present in cytoplasmic ribosomes are identical to those of the immunologically cross-reacting proteins present in the nucleolus. Nucleolar localization for a distinct ribosomal protein has also been suggested for ribosomal protein S14 by Chooi and Leiby (14). These authors have concluded, from an immunoelectron microscopic localization study on spread preparations of actively transcribed rRNA genes from *Drosophila* egg chambers and embryos, that protein S14 is among the first to associate with the 5′ end of the nascent pre-rRNA. However, in situ immunolocalization of protein S14 did not reveal a specific enrichment of this protein in the nucleolus of intact cells and was negative on cultured cells of the same species (15). In another study, monoclonal antibodies against proteins associated with ribosomes isolated from chicken liver were described by Towbin et al. (57). Although some of the antigens could be related to authentic ribosomal proteins (S6, L7, L18a), others could not (e.g., polypeptide spots designated P_0, P_1, P_2). However, these authors (57) have not mentioned nucleolar localization of the identified ribosomal proteins (an intranuclear location of ribosomal protein S6 has been concluded from microinjection experiments, reference 58). More recently, Todorov et al. (59) have examined conventional rabbit antisera against various constituent proteins of the small ribosomal subunit in immunoblot experiments, using proteins recovered in various preribosomal particle fractions. These authors have concluded that a number of ribosomal proteins are already present in preribosomal particles but they have not detected four of the ribosomal proteins tested (S2, S19, S26, and S29) in any nucleolar ribonucleoprotein fraction, which suggests that these proteins are added during or after translocation of ribosomal precursors into the cytoplasm. None of these studies has attempted to localize directly the specific antigens in situ. Human autoantibodies against ribosome-associated antigens, in some cases identified as ribosomal proteins (60), have also been used for immunolocalization, and examples of nucleolar staining (61) as well as lack of nucleolar staining (62) have been reported. However, the identity of the specific antigen(s) localized has not been established (for a review see reference 63).

Clearly, our localization data show that protein S1 does not belong to those ribosomal proteins that are absent from the nucleolus and are added only after the precursor has left the nucleolar compartment (26, 59, 64–66). Previous studies of the protein composition of fractions of various forms of nucleolar preribosomal particles have been interpreted to show that certain ribosomal proteins occur in the nucleolus and are added to precursor molecules of rRNA at different stages of RNA processing and particle maturation (59, 64–71). As it is generally assumed that different stages of pre-rRNA processing occur in different nucleolar subcompartments (for review see reference 4), this sequential appearance of ribosomal proteins might suggest that the specific ribosomal

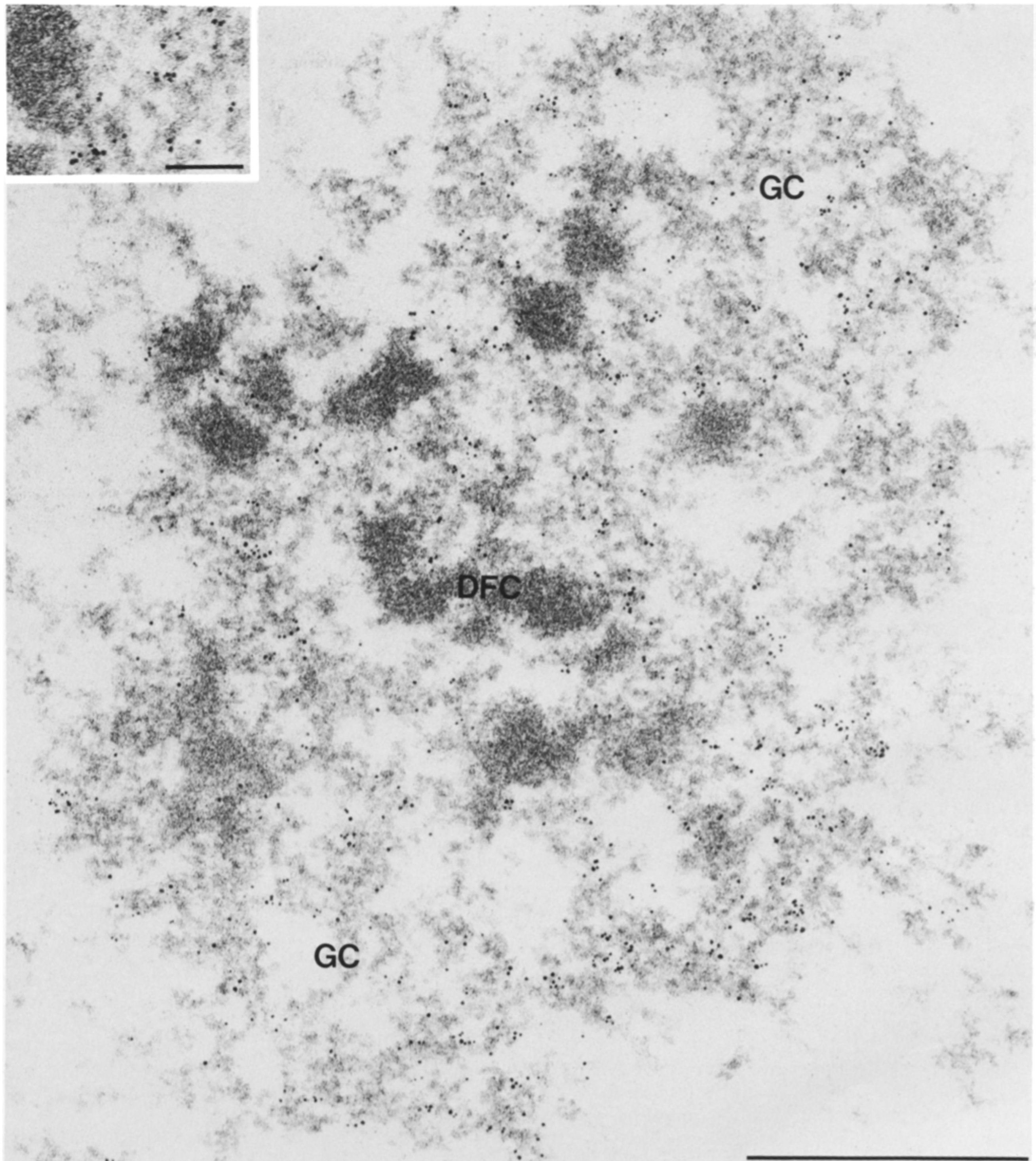


FIGURE 6 Survey electron micrograph presenting the immunolocalization of protein S1 in the nucleolus of a hepatocyte of regenerating rat liver, using monoclonal antibody RS1-105 and the immunogold label technique. The granular component (*GC*) of the nucleolus is specifically labeled with the 5-nm gold particles, whereas the dense fibrillar component (*DFC*) is not labeled (seen at higher magnification in the inset). Bars, 0.5 μm and 0.1 μm (*inset*). × 94,000 and × 133,000 (*inset*).

proteins are added in different locations of the nucleolus.

The immunolocalization of protein S1 in the granular component of the nucleolus and its absence both in the fibrillar centers and the dense fibrillar component indicate that this ribosomal protein does not associate with the nascent pre-rRNA but is added at a later stage of maturation. This means that, among the constituents of the small ribosomal subunit, protein S1 is added significantly later than protein S14 which, according to Chooi and Leiby (14), attaches early on the 5′ end of the nascent pre-rRNA. Our observations appear to be in agreement with those of Auger-Buendia and Longuet (70) who have not detected protein S1 in fractions

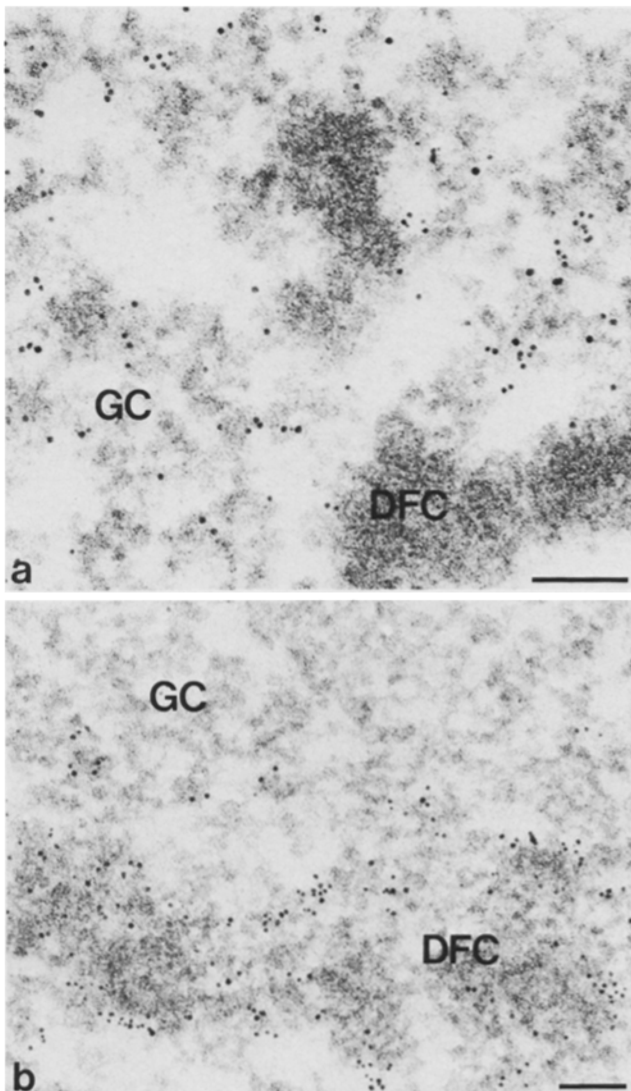


FIGURE 7 Comparison of the nucleolar localization of ribosomal protein S1 (a, rat hepatocyte) with a nucleolar protein (M_r 180,000; cf. reference 43) that is contained in the dense fibrillar component (b, *Xenopus laevis* hepatocyte). Note the distinctly different localization of both antigens: monoclonal antibody RS1-105 reacts with the granular component (*GC* in *a*) whereas monoclonal antibody No-114 decorates the dense fibrillar component (*DFC* in *b*). Bars, 0.1 μm. (*a*) × 125,000; (*b*) × 92,000.

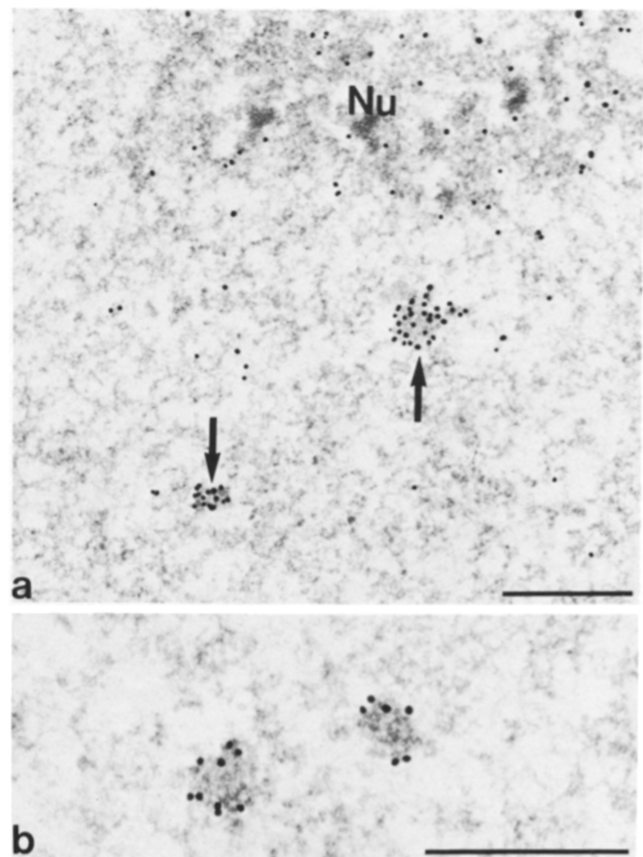


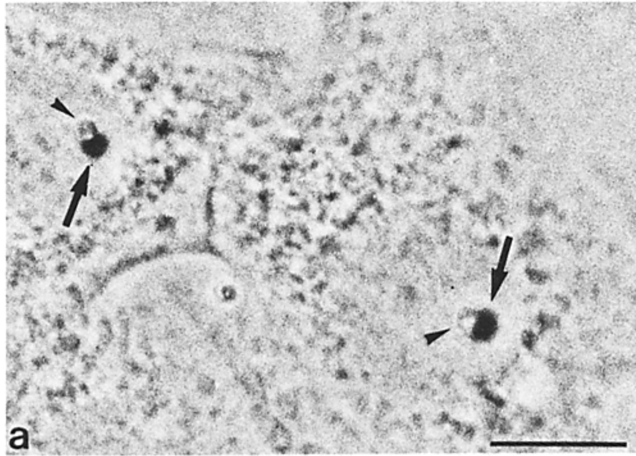
FIGURE 8 Identification of the small protein S1-containing nucleoplasmic entities described in Fig. 4 by electron microscopic immunolocalization in hepatocyte nuclei of regenerating rat liver (these specimens have been fixed with formaldehyde after incubation with the primary antibody). The survey electron micrograph (*a*) shows a part of the nucleolus (*Nu*) and two of the protein S1-containing nucleoplasmic aggregates (arrows) which are heavily labeled with gold particles of 20 nm diam. At higher magnification (*b*), these aggregates appear as distinct structural entities of relatively high electron density, most of them with diameters ranging from 0.1 to 0.3 μm, and display a finely granular composition. Bars, 0.5 μm. (*a*) × 33,000; (*b*) × 54,000.

of 80S preribosomal particles from murine leukemia cells. This suggests that protein S1 is added after cleavage of the pre-rRNA, a hypothesis that would be compatible with the location of this protein in precursor particles specific for the small ribosomal subunit that are transiently accumulated in the granular component of the nucleolus.

Although association with rRNA is the most likely form of existence of structure-bound protein S1 in the nucleolus, we cannot presently exclude the existence of some protein S1 in other, e.g., protein–protein, forms of association in a special nucleolar storage.

The intense immunolabeling of protein S1 in nucleoli and

FIGURE 9 Localization of ribosomal protein S1 in segregated nucleoli of cultured *Xenopus laevis* kidney cells (XLKE) after exposure to actinomycin D. In phase-contrast microscopy (*a* and *b*), the drug-induced segregation of nucleoli into a phase-dark (arrows) and a phase-light (arrowheads) component is clearly seen. With antibody RS1-105 (*a'*) the phase-dark hemisphere, which corresponds to the granular component of the nucleolus, is specifically stained. For comparison, the localization of the nucleolar M_r 180,000 protein is shown in *b'* to be confined to the phase-light hemisphere, i.e., the fibrillar component (arrowheads). (*c*) Electron micrograph of conventionally fixed, actinomycin D-treated XLKE cells reveals the characteristic segregation of the nucleolar structure into the dense fibrillar component (*DFC*), the granular component (*GC*), and a caplike structure closely apposed to the dense fibrillar component (probably containing the fibrillar centers). (*d*) Immunoelectron microscopy using antibody RS1-105 shows strong reaction exclusively with the granular component (*GC*) of the segregated nucleoli, as indicated by the distribution of the 5-nm gold particles. Bars, 10 μm (*a* and *b*), 0.5 μm (*c*), and 0.2 μm (*d*). (*a* and *b*) × 1,800; (*c*) × 15,000; (*d*) × 157,000.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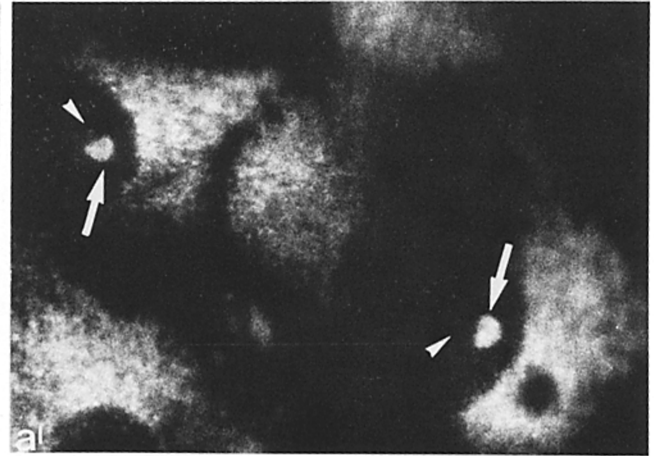
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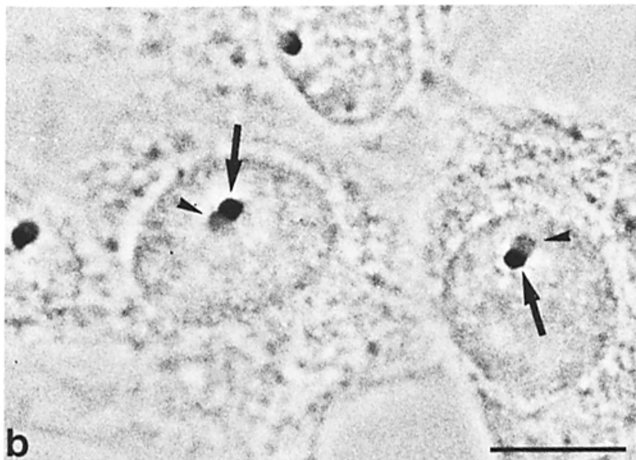
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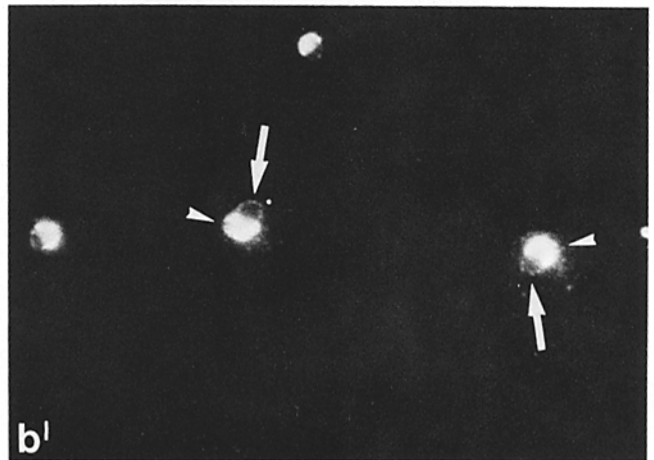
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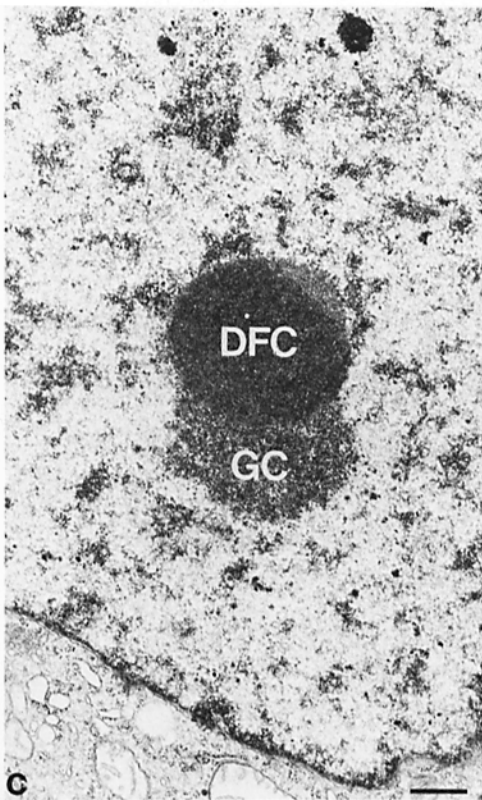
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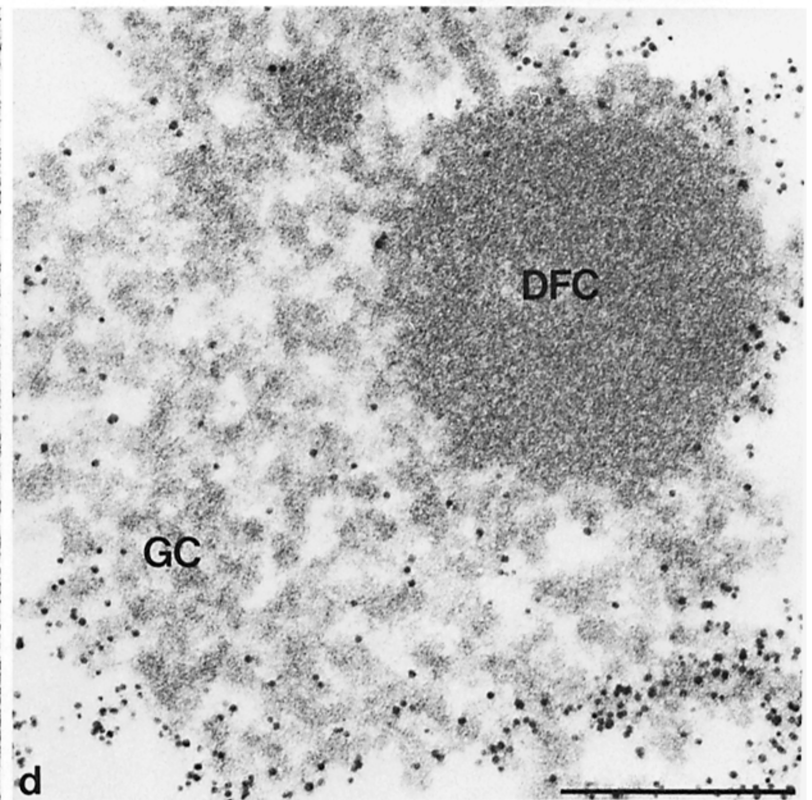
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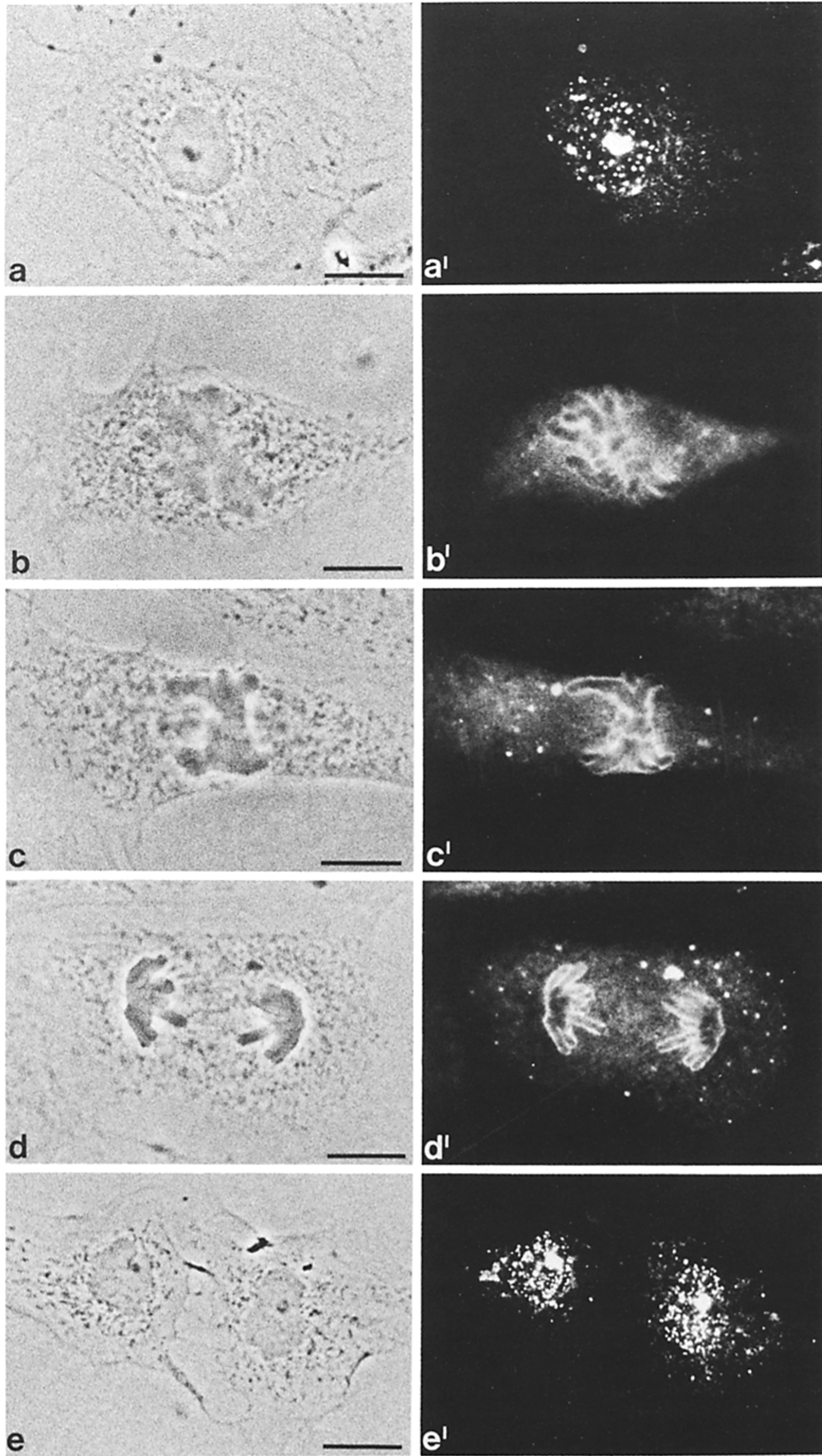
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cytoplasmic ribosomes is contrasted by an absence of reaction in other parts of the nucleus, with the exception of the small nucleoplasmic granules (Figs. 3, 4, and 8) that may represent forms of nucleocytoplasmic translocation of precursors to the small ribosomal subunit. While this lack of reaction in most of the nucleoplasm could be artificially induced by losses of a soluble form of the antigen during the incubation steps of the immunolocalization, the significance of the finding is supported by controls that minimize such losses and by our ability to stain soluble nucleoplasmic proteins by the methods used (data not shown; cf. reference 72). Therefore, we think that the absence of antigen in most of the non-nucleolar part of the nuclear interior is best explained by a very small nucleoplasmic pool of precursor particles to the small ribosomal subunit, an interpretation that is in agreement with various biochemical reports (23, 73–75). In contrast, Warner (66) has concluded, from fractionation studies of HeLa cells, that quite sizeable nucleoplasmic pools of various proteins of the small ribosomal subunit exist. However, in his study he did not rigorously exclude the possibility that some proteins recovered in the "nucleoplasmic fraction" might have been artificially released from nucleolar material during the fractionation.

The distribution during mitosis of the structures containing nucleolar ribosomal protein S1 is remarkable for its difference from that of other nucleolar proteins. For example, RNA polymerase I remains attached, to a considerable degree, to the NOR of the metaphase chromosomes (8), in this respect similar to the M_r 110,000 phosphoprotein C23 (19), whereas the M_r 180,000 protein constituent of the dense fibrillar component dissociates and disperses over the cytoplasm of the metaphase cell (43). In contrast, protein S1 is recognized, during prophase and prometaphase, in light microscopically detectable granules scattered throughout the nucleoplasm as well as in the residual nucleolar body. This suggests that during early steps of nucleolar disintegration, portions of the fragmented granular component disperse over the nucleoplasm and later, after breakage of the nuclear envelope, also over the cell plasm. During metaphase and anaphase, antibody-labeled protein S1 appears to be enriched on the surfaces of the chromosomes in form of a distinct, uniformly fluorescent perichromosomal coat. This pattern of distribution is somewhat reminiscent of that described for the nonribosomal M_r 37,000 phosphoprotein B23 which has been reported to be concentrated in both the fibrillar and the granular components of the nucleolus during interphase (17, 20, 22). During telophase and early interphase, the protein S1-containing material reassembles into distinct small granules accumulating within the confinements of the reformed nuclear envelopes. Finally, this material is gradually recovered in the reconstituting nucleoli. This dissociation and reassembly cycle shows that the nucleolar form of protein S1-containing material is different from cytoplasmic ribosomes as it is selectively recollected into the daughter nuclei, indicative of the existence of a sorting process separating the nucleolar S1 complexes from cytoplasmic ribosomes. Our immunocytochemical observations are compatible with the conclusions of Fan and Penman (76), based on biochemical studies of nucleolar RNA, that ribosomal precursor material released from disintegrated nucleoli persists during mitosis and repopulates the postmitotic nucleolus (77, 78). The ultrastructural organization of the small nucleolus-derived granules and the chromosome-associated S1 material in mitotic cells is currently being studied in our laboratory by immunoelectron microscopy.

Our results also support the concept that the acidic protein S1 is a true ribosomal component of different species (for discussion of the significance of protein S1 as a ribosomal constituent see references 46 and 48). Whether this component of the eucaryotic ribosome is chemically and functionally related to the procaryotic ribosomal protein S1 involved in the binding of mRNA to the small ribosomal subunit (79, 80) remains to be seen in future experiments.

This paper is part of a series aiming at the differential "mapping" of nucleolar proteins, ribosomal ones included, in relation to the nucleolar subcompartments that so far have been defined primarily by morphological criteria only. We hope that the elucidation of the topological arrangement of nucleolar components will promote our understanding of the functional organization of this structure and of the early steps of ribosomal assembly in particular.

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FIGURE 10 Distribution of ribosomal protein S1 in PtK2-cells during mitosis as visualized by immunofluorescence microscopy using antibody RS1-105. Phase-contrast images are shown in *a–e*, and the corresponding immunofluorescence micrographs are shown in *a′–e′*. In early mitotic prophase (*a* and *a′*), protein S1 is localized in the still largely compact nucleolus and in numerous small nucleoplasmic granules that probably include fragments of the disintegrating nucleolus. In addition, diffuse cytoplasmic staining, probably reflecting ribosomes, is seen. In prometaphase (*b* and *b′*), metaphase (*c* and *c′*), and anaphase (*d* and *d′*), a strong fluorescence on chromosomal surfaces is noted beside a diffuse fluorescence in the perichromosomal cell plasm. In late telophase (*e* and *e′*) the antibody stains the reconstituting nucleoli as well as several granular entities within the confinements of the forming daughter nuclei, and some more diffuse fluorescence in the cytoplasm. Bars, 10 μm. × 1,250.

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